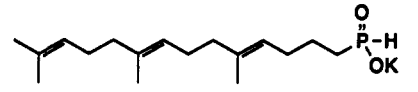
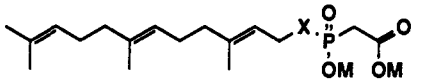
cytes, whereas PMP 3 was inactive. Both decreasing and increasing the length of the $(CH_2)_n$ linker between the isoprenyl subunit and the diphosphate surrogate (**4a**, n = 2; **4c**, n = 4) led to a loss of potency in the enzyme assay. Introduction of an oxygen atom in the linker in the form of a phosphorus ester (**5a** and **5b**) resulted in more active enzyme inhibitors than the corresponding C-linked isosteres (**4b** and **4c**, respectively). The optimal overall chain length of both the C- and O-linked inhibitor series (**4b** and **5a**) corresponds to that which is isosteric to FPP. Phosphinylformate **5a** (I_{50} = 8.7 μM), the most potent enzyme inhibitor in this study, also exhibited commensurate activity in the rat hepatocyte assay (I_{50} = 6 μM).

The inhibition of microsomal squalene synthetase by **5a** (0–30 μM) was studied at increasing FPP (5–50 μM) and saturating NADPH (0.9 mM) concentrations. Double-reciprocal analysis of the kinetic data (Figure 1) indicates that **5a** is a competitive inhibitor with respect to FPP (K_i = 2.6 μM). In comparison, the K_i of **3** is 10 μM and the apparent K_m of FPP is 12.7 μM.[1,3a]

Carboxylate esters **4d** and **5d** are weak inhibitors of squalene synthetase compared to the corresponding salts **4b** and **5a**, but the salts and esters exhibited similar activity in the hepatocyte assay. We speculate that **4d** and **5d** serve as effective precursors to **4b** and **5a** in whole cells but not in the enzyme assay. In addition, **4d** and **5d** inhibited the conversion of [^{14}C]acetate to cholesterol in human skin fibroblasts under conditions where the salts are inactive, suggesting that the lipophilic esters can more readily enter this cell type.[15]



6



7a, X = CH_2, M = K
7b, X = O, M = Li

Phosphinylformates are unstable at low pH,[16] undergoing decarboxylation to the corresponding phosphonous acids (e.g. **4b** to **6**). We prepared **6** independently via the acid-catalyzed hydrolysis of **9b** to **14b** (78% from 8, n = 3, X = I) followed by treatment with KOH.[9] This material was found to be identical with that which is obtained upon reaction of **4b** with aqueous HCl. Phosphonous acid **6** is 6.2-fold less potent than **4b** in the squalene synthetase assay, suggesting that the activity of **4b** is not due to its conversion to **6**.

The phosphinylacetate isosteres **7a** and **7b**,[17] based on the related antiviral agent PAA,[6] are less potent squalene synthetase inhibitors than the corresponding phosphinylformates of the same chain length (**4b** and **5a**). For comparison, PAA is inactive in the squalene synthetase assay at up to 1 mM.

In summary, we have demonstrated that the phosphinylformate moiety is a novel, dianionic diphosphate surrogate for the construction of inhibitors of squalene synthetase. In addition, isoprenyl phosphinylformates and their esters are the first inhibitors of this enzyme to block the synthesis of cholesterol in whole cells. We anticipate that the phosphinylformate function, and the chemistry described herein, will find utility in the synthesis of analogues of other diphosphate-containing molecules of biological significance.[13,19]

Acknowledgment. We would like to thank the Bristol-Myers Squibb Analytical Chemistry Department for obtaining elemental analyses, mass spectra, IR spectra, and certain NMR spectra. In addition, we would like to acknowledge helpful discussions with Drs. Donald S. Karanewsky, William H. Koster, and William A. Scott.

Supplementary Material Available: Representative procedures for the synthesis of **4a** and **5a** are available (2 pages). Ordering information is given on any current masthead page.

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(17) **7a** was prepared from phosphonyl chloride **5a** of ref 3b: (a) $LiCH_2CO_2$-*t*-Bu, THF, -78 °C; (b) KOH. **7b** was prepared by from farnesyl chloride: (a) $(n\text{-}Bu_4N^+)_2[O_3PCH_2CO_2Et]^{2-}$, CH_3CN;[18] (b) AG 50W-X8 (Li form); (c) LiOH.
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Targeting Renal Dipeptidase (Dehydropeptidase I) for Inactivation by Mechanism-Based Inactivators

Renal dipeptidase (dehydropeptidase I, EC 3.4.13.11) is a zinc-containing hydrolytic enzyme that shows preference for dipeptide substrates with dehydro amino acids (e.g., 2-amidoacrylic acid derivatives) at the carboxy position; however it can accommodate substrates with both D or L amino acids at that position as well.[1] While the physiological function of this enzyme is not known presently, it has been implicated in metabolism of glutathione and its derivatives.[2] In addition, it is responsible for the hydrolytic scission of the lactam bond in carbapenems, potent broad-spectrum antibiotics that are resistant to the action of microbial β-lactamases.[3] Enzymic turnover of carbapenems in vivo poses a serious obstacle to clinical efficacy of these bactericidal agents; therefore specific inhibitors for this enzyme are widely sought.[4]

We have reported recently on the ability of carboxypeptidase A, a prototypic zinc protease, to carry out a deprotonation reaction that was exploited in inactivation of the enzyme.[5,6] We report here our studies of the first two mechanism-based inactivators designed specifically for porcine renal dipeptidase, which take advantage of the

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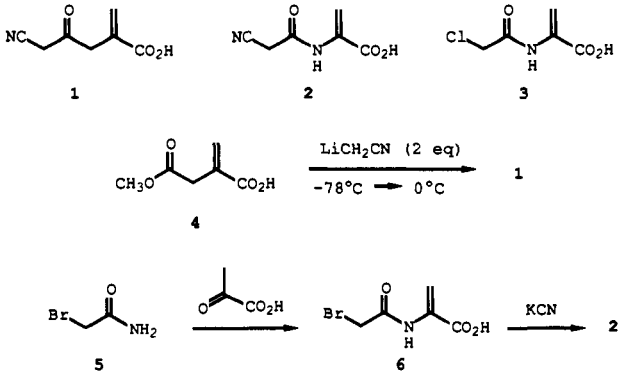
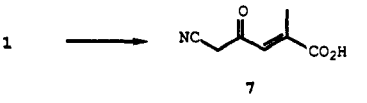
same type of reaction reported for carboxypeptidase A. The deprotonation/enolization may be a general reaction common to zinc proteases, which could be conscripted in the design of highly specific mechanism-based inactivators for this family of enzymes.

Compounds 1 and 2 were prepared as analogues of the renal dipeptidase substrate *N*-(chloroacetyl)dehydroalanine (3).[1] The target molecules were each synthesized in two steps. Reaction of compound 4, prepared from itaconic anhydride according to a literature method,[7] with the lithium salt of acetonitrile at −78 °C afforded 1 in 70% yield. Bromoacetamide (5) was allowed to react with pyruvic acid under reflux in toluene to give 6 in 59%. A facile displacement of the bromo group with cyanide took place by stirring 6 and KCN in methanol to yield 2 in 65%.[8]



The porcine enzyme was purified to homogeneity in a single affinity chromatographic step by a modification of a literature method.[9] From two kidneys 2.7 mg of the protein was purified, which showed a specific activity of 9.14 μmol min^{-1} mg^{-1}, when assayed with glycinyldehydrophenylalanine (Gly-dPhe).[10] Both compounds 1 and 2 inactivated the enzyme in a time-dependent manner. Enzyme inactivation showed saturation, followed pseudo-first-order kinetics, was inhibited by the substrate Gly-dPhe and was irreversible as extensive dialysis over 4 days did not regenerate any activity. A double-reciprocal analysis of the pseudo-first-order inactivation rates versus the inhibitor concentrations furnished the values for k_{inact} of 0.151 ± 0.012 min^{-1} and 0.277 ± 0.024 min^{-1} and K_m of 10.30 ± 0.78 mM and 0.416 ± 0.036 mM for 1 and 2, respectively. Partition ratios (k_{cat}/k_{inact}), measured according to the titration method,[11] were 217 ± 37 and 55 ± 2 for the ketonic and the peptidic molecules, respectively. In turn, k_{cat} values of 33 ± 9 min^{-1} and 15 ± 2 min^{-1} were estimated for inactivators 1 and 2, respectively.[12]

Pyruvate formation from 2 was detected by HPLC analysis. Further, conversion of pyruvate to L-lactate by lactate dehydrogenase, as monitored by the method of Borgman et al.,[13] allowed for an estimation of the pyruvate concentration that was consistent within 10% of the value expected from the partition ratio for this compound. Attempts to characterize the turnover product for compound 1 have thus far been unsuccessful. A possibility exists for a competing deprotonation at the α position to the carbonyl, proximal to the carboxylate in 1. Such a deprotonation may give rise to compound 7, an α,β-unsaturated ketone, which would be prone to polymerization. Compound 1 exists as an α,β-unsaturated carboxylate under the same conditions, and is stable in solution for several hours. Deprotonation/enolization of ketonic inhibitors for carboxypeptidase A[14] and angiotensin-converting enzyme[15] at a comparable position is precedented.



The following experiments shed light on the inactivation process. (i) Despite the presence of the activated α-bromoacetyl and the α,β-unsaturated functions, compound 6 served only as a substrate for renal dipeptidase (K_m = 0.74 mM, k_{cat} = 32.3 min^{-1}); no inactivation due to potential displacement of the bromo group or Michael addition was noted.[16] (ii) A radioactive form of 2 was prepared where ^{14}C was incorporated at the nitrile position (specific activity was 27 000 dpm/nmol). A 0.5-mg portion of the enzyme was inactivated with the labeled compound 2, followed by extensive dialysis. The inactivated enzyme was incorporated with 1.09 equiv of the inactivator per

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(8) Spectral data for 1: ^1H NMR ($CDCl_3$) δ 6.54 (1 H, s), 5.90 (1 H, s), 3.60 (2 H, s), 3.57 (2 H, s); IR (mineral oil) 3200, 2260, 1700, 950 cm^{-1}; EI MS 153.0423 (exp 153.0425). Spectral data for 2: ^1H NMR (DMSO-d_6) δ 9.56 (1 H, s), 6.28 (1 H, s), 5.76 (1 H, s), 3.94 (2 H, s); IR (mineral oil) 3328, 2284, 1715, 1688, 939 cm^{-1}, CI MS 155 (M + H). Both compounds were pure as judged by their HPLC trace on an analytical ODS column. Intermediates 4 and 6 were also characterized by NMR, IR, and MS.

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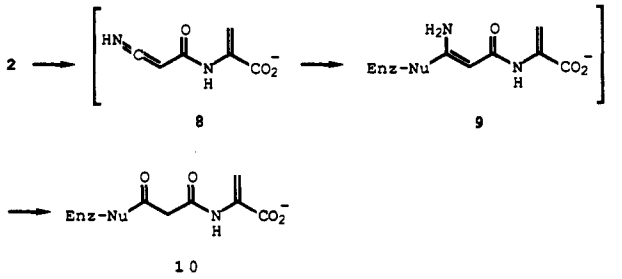
(16) This finding is consistent with the literature report that *N*-(chloroacetyl)dehydroalanine is a fair substrate for dehydropeptidase I (see ref 1).

protein monomer.[17] (iii) Upon denaturation of the inactivated enzyme in 7 M urea all of the radioactivity remained associated with the protein. Furthermore, the enzyme inactivated by the radioactive form of **2** was allowed to incubate with 1 M H_2NOH under denaturing conditions (7 M urea, pH 8.0) for 5 h. Under these conditions, none of the radioactivity was liberated from the protein, suggesting that the modified amino acid may not be an acidic residue. These experiments document the covalent nature of protein modification and argue for the high specificity of such interaction.

We propose the following plausible mechanism for the action of the inactivators. Compounds **1** and **2** undergo an enzyme-mediated deprotonation α to the carbonyl function,[18] conceivably by the zinc-bound water/hydroxide.[19] The deprotonation step is expected to lead to the formation of a ketenimine, followed by the trapping of an active site nucleophile, resulting in covalent modification of the enzyme. Our attempts at detecting the transient appearance of a new chromophore at 270–300 nm[20] from **2** during the course of inactivation were not successful. It appears that after nucleophile capture (**8** → **9**), the resultant enamine (**9**) undergoes facile hydrolysis to a species such as **10**, for which the enol form is not expected to predominate.[21]



Registry No. 1, 133648-14-3; 2, 133626-26-3; 5, 683-57-8; 6, 133626-27-4; H_3CCOCO_2H, 127-17-3; dipeptidase, 9031-99-6.

(17) The chromophore for the α,β-unsaturated system of **2** (ϵ_{235} ~4400 M^{-1} cm^{-1}) was incorporated into the inactivated protein. On the basis of the extinction coefficient of **2** in solution, approximately 2.7 molecules of the inactivator were appended to the protein. In light of the caveat that large changes in the extinction coefficient of the chromophore in the active site may be expected, this type of analysis may carry a large inherent error. The extent of protein modification by the radioactivity measurements should be more reliable.

(18) Such a rearrangement for nitrile has been exploited in enzyme inactivation previously: Miles, E. W. Effects of Modification of the β-2 Subunit of the $\alpha2\beta2$ Complex of Tryptophan Synthase by α-Cyanoglycin, a Substrate Analog. *Biochem. Biophys. Res. Commun.* **1975**, *64*, 248–255. Maycock, A. L.; Suva, R. H.; Abeles, R. H. Novel Inactivators of Plasma Amine Oxidase *J. Am. Chem. Soc.* **1975**, *97*, 5613–5614. Tang, S. S.; Trackman, P. C.; Kagan, H. M. Reactions of Aortic Lysyl Oxidase with β-Aminopropionitrile. *J. Biol. Chem.* **1983**, *258*, 4331–4338.

(19) For a discussion of the mechanistic role proposed for the metal-bound water in zinc proteases, see: Mathews, B. W. Structural Basis of the Action of Thermolysin and Related Zinc Peptidases. *Acc. Chem. Res.* **1988**, *21*, 333–340. Christianson, D. W.; Lipscomb, W. N. Carboxypeptidase A. *Acc. Chem. Res.* **1989**, *22*, 62–69.

(20) For a survey of chromophores typical for β-amino-α,β-unsaturated carbonyl compounds, consult: Ostercamp, D. L. Vinylogous Imides. 2. Ultraviolet Spectra and the Application of Woodward's Rules. *J. Org. Chem.* **1970**, *35*, 1632–1641.

(21) The pK_a for the α-methylene in structures such as **10** is approximately in the range 14–16.

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Book Reviews

Nicotine Psychopharmacology. Molecular, Cellular, and Behavioural Aspects. Edited by S. Wonnacott, M. A. H. Russell, and I. P. Stolerman. Oxford University Press, Oxford and New York. 1990. XIX + 427 pp. 16 × 24 cm. ISBN 0-19-261614-5. $80.00.

This book aims "to bring together a very wide range of material from different areas of research relevant to understanding the psychopharmacology of nicotine". In 11 relatively free-standing chapters, experts review the pharmacokinetics, metabolism, and pharmacodynamics of nicotine and its effects on animal and human behavior and on synaptic transmission in the nervous system (studied with electrophysiological, biochemical, or molecular biology techniques). The role of nicotine in tobacco dependence is reviewed from different angles in three chapters, and two more deal with the possible relevance of nicotinic cholinergic mechanisms to Parkinson's and Alzheimer's disease.

Tight editorial control has made the book laudably cohesive notwithstanding the diversity of subjects and the large number of authors. Nonetheless, some contributors appear innocent of recent developments in related fields and for instance stick to older pharmacologic classifications in spite of the demonstration by molecular cloning of a much greater diversity in neural nicotinic receptors. Most chapters follow a uniform pattern, beginning with an introduction that outlines the scope and the topics to be discussed. This is followed by the body of the chapter, clearly subdivided with the progressive decimal numbering system in headings and subheadings (all titles of which can be found again in an extensive analytical table of contents). The last section of each chapter summarizes the evidence and presents the conclusions. The chapters are largely self-contained, but well-integrated, with extensive and detailed cross-referencing, e.g. to specific tables or figures in chapters by other contributors. Occasional overlap was probably unavoidable, and it is rarely excessive except for the last chapter Nicotine Intake and Its Control Over Smoking which reiterates much that has been dealt with in the first two chapters, as can be surmised already from their titles: "Tobacco smoking and nicotine dependence" and "Nicotine dependence: animal studies".

Most chapters review the (often vast) literature expertly and critically, interpreting for the nonspecialist reader the shortcomings as well as the merits of certain studies, alerting him/her to some methodological pitfalls, and discussing apparent and real discrepancies. At times the historical development in a field is sketched, affording better appreciation for the context of earlier work and for changes of opinion over time. Gaps in our current knowledge are honestly indicated, even when they undermine entire areas of research: the relevance for tobacco smoking of studies using intravenous, percutaneous, or oral administration of nicotine is called into question as none of these routes probably mimics the repeat bolus dosage of cigarette puffs. Although most authors manage reasonably well to avoid jargon, they largely fail to keep the editors' promise of providing "descriptions and ex-